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13. ABSTRACT *(Maximum 200 words)*

This report describes recent results of studies designed to investigate the metabolic effects of perfluorocarboxylic acids on liver carbohydrate and high-energy phosphorous metabolism. Carbon-13 nuclear magnetic resonance (NMR) spectroscopy was used in conjunction with ^{13}C isotope labeling to monitor the dynamic conversion of glucose to glycogen in rat liver *in vivo*. The results show that perfluorodecanoic acid (PFDA) causes a marked inhibition in hepatic glycogen synthesis in rats at 3 days post treatment ($n = 5$) and complete inhibition at 5 days post dose ($n = 5$). Hepatic glucose and blood glucose levels are also slightly elevated within the first 15 min. following a glucose load in PFDA rats versus controls ($p < 0.05$). Preliminary data reveal that glycogen synthesis from alanine via gluconeogenesis is functional in PFDA-treated rats. This suggests that the inhibition in glycogenesis from glucose may involve the transport of glucose into hepatocytes and/or its phosphorylation by glucokinase. Further studies are in progress which investigate this hypothesis. In studies of the high-energy phosphorous metabolism in PFDA-treated rats, ^{31}P NMR reveals normal ATP levels and an anomalous signal in the phosphomonoester region of the liver spectrum. The source of this phosphorous metabolite has not yet been identified and is the focus of ongoing research efforts. These studies are providing new information about the metabolic effects of perfluorocarboxylic acids and advancing the development of NMR techniques in the field of toxicology.

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ANNUAL TECHNICAL REPORT

Title: Hepatic Metabolism of Perfluorinated Carboxylic Acids and Polychlorotrifluoroethylene: A Nuclear Magnetic Resonance Investigation *in Vivo*

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Annual Technical Report

Introduction

The primary objective of this study is to gain a better understanding of the toxicological mechanisms associated with certain halocarbon compounds. Specifically, the focus of our research has concerned the metabolism of perfluorocarboxylic acids and the effects they have on endogenous liver metabolism. These studies involve the application of nuclear magnetic resonance (NMR) spectroscopy *in vivo* and strive to further our understanding of toxicological mechanism and expand the applicability of the NMR technique in the field of toxicology.

In the previous annual technical report (submitted December 15, 1990) we described ^{19}F NMR experiments which were designed to investigate the metabolism of perfluorooctanoic acid (PFOA) and perfluorodecanoic acid (PFDA) in rats. These studies are now complete and a manuscript was submitted to *Chemical Research in Toxicology*. (A copy of this manuscript was also sent to Lt. Col. J. Cervany, AFOSR.) The reviews concerning this manuscript have recently been received. The manuscript is currently being revised in accordance with the referees' comments and should be acceptable for publication pending these revisions.

This report will summarize our research efforts and progress since the previous technical report. This will consist of a very brief mention of the ^{19}F NMR work in relation to one new piece of information. Mainly, the report will concentrate on our efforts to investigate the effects of PFOA and PFDA on liver carbohydrate and high-energy phosphate metabolism *in vivo*.

I. ^{19}F NMR Studies

The studies involving a ^{19}F NMR investigation of PFOA and PFDA in rat liver *in vivo* and various bodily fluids has been described previously and will not be reiterated here. There is, however, one new piece of information relating to this particular work. In the last annual technical report we mentioned that the ^{19}F NMR spectrum of urine from PFDA- and PFOA-treated rats showed a peak which was not attributable to the parent compound. Since that report, this NMR signal has been identified as the resonance of inorganic fluoride. In addition, spectra of urine from control rats were found to contain this same resonance, and selective ion electrode measurements of [F^-] yield similar concentrations in urine from both treated and control rats. Thus, it is concluded that this fluoride ion is not a result of any defluorination of the perfluorocarboxylic acids, but is rather from a dietary source.

II. Glucose and Glycogen Metabolism

The effects of PFOA and PFDA treatment on hepatic carbohydrate metabolism are being investigated by proton-decoupled ^{13}C NMR spectroscopy. Preliminary data was presented in the previous technical annual report. In fact, the methods concerning this study were described in detail in the previous report and, therefore, only a brief account of the methods will be given here. This report will focus primarily on the results and our progress in this area.

Proton-decoupled ^{13}C NMR liver spectra were acquired with 5 minute time resolution from rats *in vivo* at 3 and 5 days post-treatment with PFDA or vehicle (control). Following the accumulation of baseline spectra, rats were given an iv. bolus of [1-^{13}C] glucose (99 atom%) via a femoral vein. Incorporation of the ^{13}C label from glucose into glycogen is easily discernible in the NMR spectrum.

Figure 1A is the ^{13}C liver spectrum at 8.5 T from a control rat at 5 days post treatment with vehicle solution (propylene glycol/water, 1:1 v/v). This spectrum was acquired 20 minutes post injection with [1-^{13}C] glucose (600 mg/kg). Signals appearing in three distinct regions are characteristic of the ^{13}C spectrum of most tissue types. The low-frequency region of overlapping signals between 15 and 50 ppm is due to the methyl and methylene carbons primarily from triacylglycerols and phospholipids. The peak at 54.6 ppm arises from the methyl carbons of the choline head groups of phospholipids and serves as an internal chemical shift reference. The two regions appearing at higher frequency constitute the single- and double-bonded allylic carbons associated with unsaturated fatty acids at ca. 130 ppm and the carbonyl carbons at ca. 172 ppm.

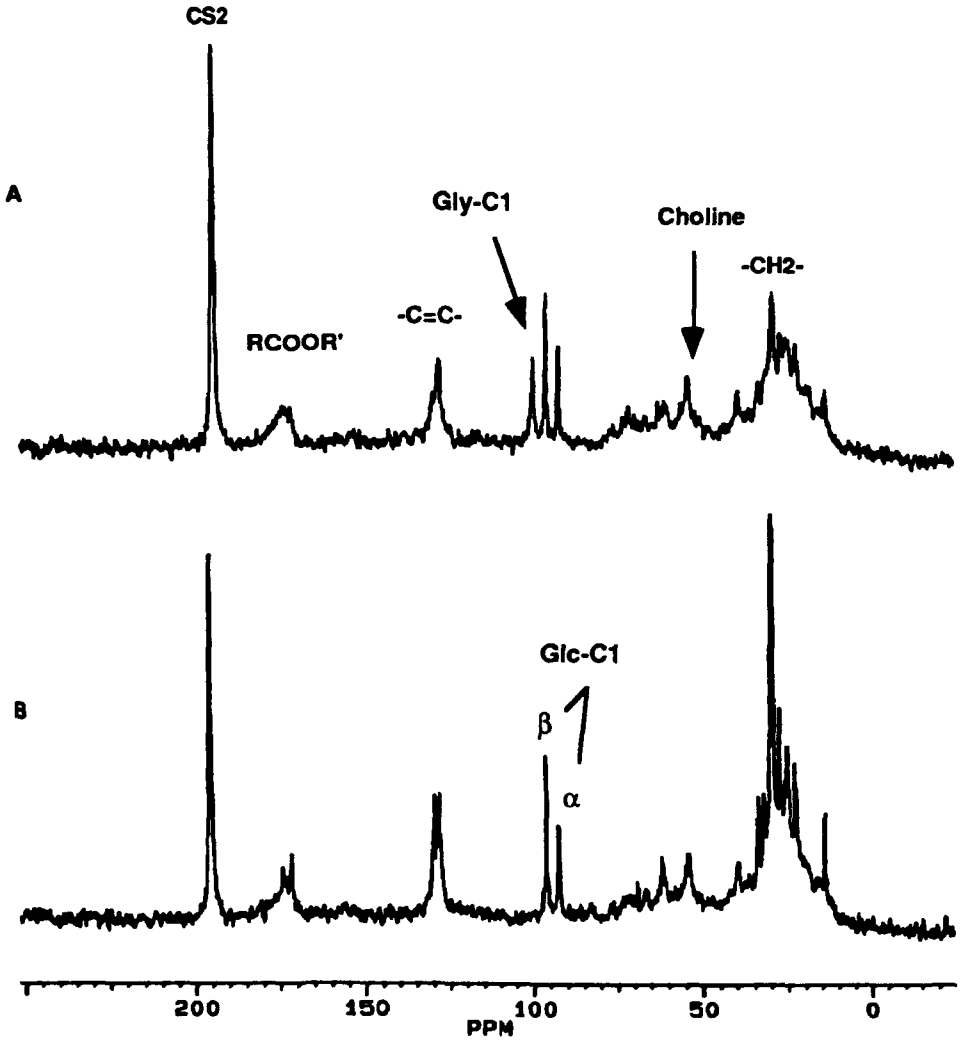

Figure 1: Proton-decoupled surface coil ^{13}C liver spectra at 8.5 T from a normal pair-fed control rat (A) and PFDA-treated rat (B) at 5 days post dose. Data were acquired using a 16 μsec pulse (@ 100W), sweep width of 25 kHz, and interpulse delay of 0.137 sec. FID's were processed using an exponential filter producing a 30 Hz line-broadening. Spectra represent 10 min. of signal averaging and were initiated at 20 min. following a bolus iv. injection of [1-^{13}C] glucose. Both glucose and glycogen signals are evident in the control animal, but only the C1-β and C1-α carbons of glucose are seen in the PFDA-treated rat. Chemical shifts are relative to TMS via the choline signal which is set at 54.6 ppm.

The peak at 196 ppm is from a CS_2 reference contained in a capillary tube positioned on the opposite side of the observation coil from the liver. Resonances between 60 and 100 ppm represent the carbohydrate region of the spectrum. The two signals at 96 and 92 ppm are the β- and α- anomers of the C1 carbon atom of glucose, respectively, and arise from the [1-^{13}C] glucose. The C1 carbon atom of the glycogen molecule resonates at 100 ppm and arises from the incorporation of the [1-^{13}C] glucose into hepatic glycogen. Thus, this spectrum clearly demonstrates that glycogen synthesis is active in control animals and the conversion of glucose into glycogen can be monitored in real time.

Figure 1B represents a ^{13}C NMR spectrum from a rat at 5 days post-treatment with PFDA and was also acquired 20 minutes post dose with [1-^{13}C] glucose. In contrast to the control animal, this spectrum reveals the absence of a glycogen peak at 100 ppm, and demonstrates that the incorporation of [1-^{13}C] glucose into glycogen is inhibited in PFDA-treated rats. In general, PFDA treatment caused a marked inhibition in glycogenesis at 3 days post dose (n=5) and complete inhibition at 5 days post dose (n=5).

The signal intensity of the C1-β glucose resonance can also be used to monitor hepatic glucose utilization. As shown in Fig. 2A, day 3 PFDA rats show a significantly higher mean liver glucose intensity (mean ± SEM) at 7.5 and 12.5 min post-glucose (87.61 ± 2.43 and 63.97 ± 2.47), as compared to control rats (73.41 ± 2.76 and 55.00 ± 2.86) ($p < 0.045$). At day 5, PFDA rats show a significantly higher mean glucose intensity ($p < 0.013$) at 7.5, 12.5, and 17.5 min post-glucose (87.97 ± 2.58, 65.53 ± 1.51, and 51.74 ± 2.62) as compared to control rats (72.07 ± 2.35, 55.20 ± 2.28, and 43.22 ± 1.36) (Fig. 2B).

Corresponding serum glucose determinations indicate no significant difference between PFDA and control groups at day 3 post treatment (Fig. 3A). However, at day 5, PFDA rats show a significantly higher blood glucose level ($p < 0.02$) as compared to control rats at 15 minutes post-glucose dose (Fig. 3B). Terminal urine glucose levels were found to be elevated in both groups; however, there was no significant difference between the two groups ($p < 0.05$).

An interesting feature observed in the ^{13}C NMR spectra of all PFDA-treated rats is an increase in spectral resolution as compared to their pair-fed controls (Fig. 1). The spectral resonances most affected are those from 15 to 50 ppm and the allylic carbons are 130 ppm which are attributed to triacylglycerides and membrane components. Note the larger intensity of the methylene signal at 30 ppm (Fig. 1B) which is mostly due to a narrowing of the NMR line. This result is most probably due to an increase in liver triglycerides which is a known effect of PFDA treatment; however, it is also possible that this may reflect an increase in membrane fluidity. This hypothesis is further corroborated by the ^{31}P NMR spectrum of liver which shows an intense signal from a phosphomonoester metabolite which may be indicative of enhanced membrane synthesis (*vide infra*).

These data suggests that PFDA significantly impacts hepatic carbohydrate metabolism, which may contribute to the overall "wasting toxicity" associated with exposure to this compound. In conjunction with lowering the rate of hepatic glucose utilization, PFDA severely inhibits hepatic glycogen synthesis. A new series of experiments are currently in progress which will provide additional information concerning the metabolic site of this inhibition. These experiments are briefly outlined below.

Figure 2

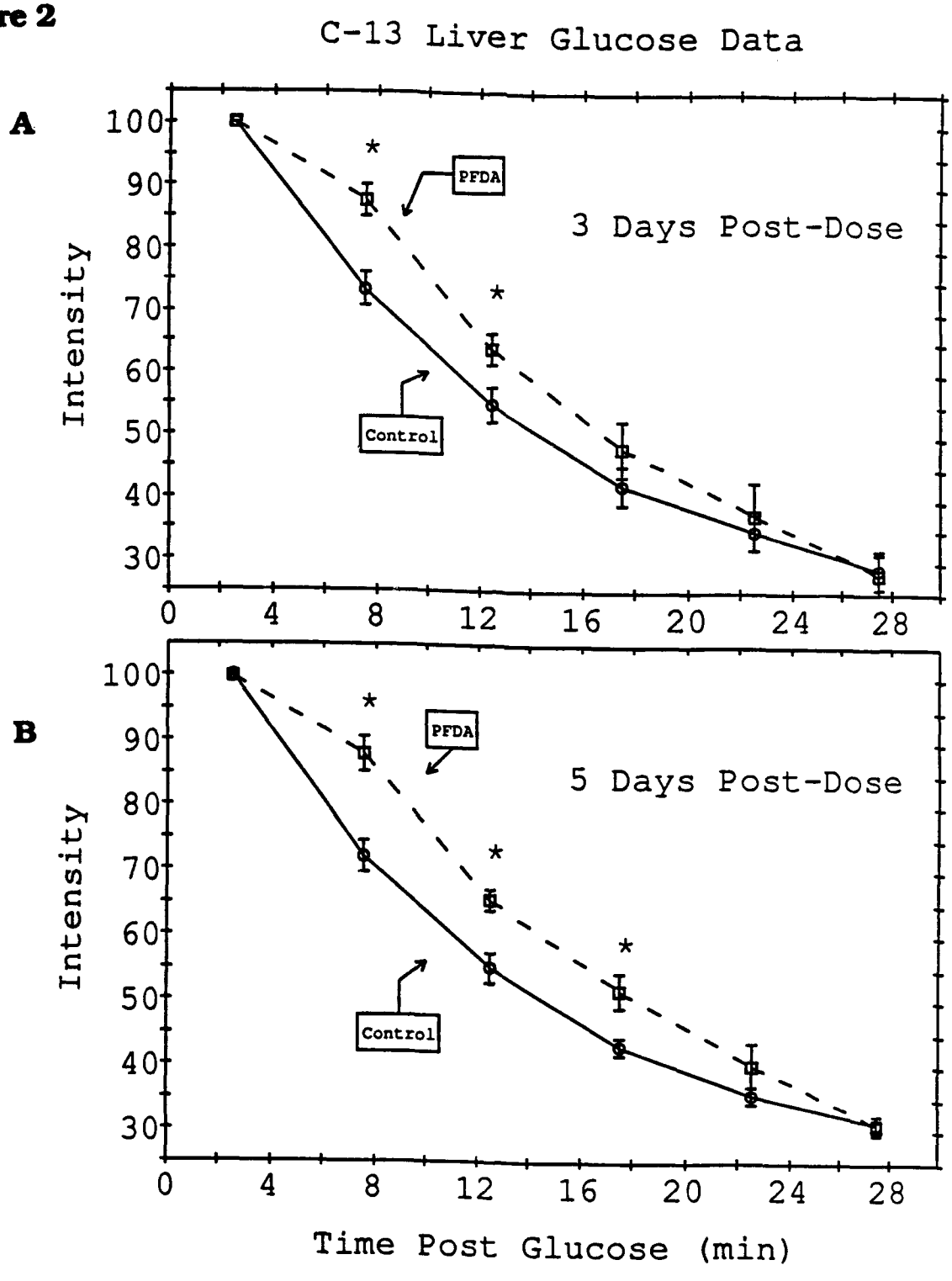

Figure 2: Represents the percent change in the NMR intensity (mean ± SEM) of the C_1 β-anomer of glucose following an i.v. injection of [1-^{13}C] glucose. In all animals, the C_1 β-signal was normalized to 100% in the first spectrum acquired post-glucose. A) Control and PFDA groups at day 3 post treatment (n = 5/group). B) Control (n = 5) and PFDA (n = 4) groups at day 5 post treatment. *Indicates a significant difference from control.

Figure 3

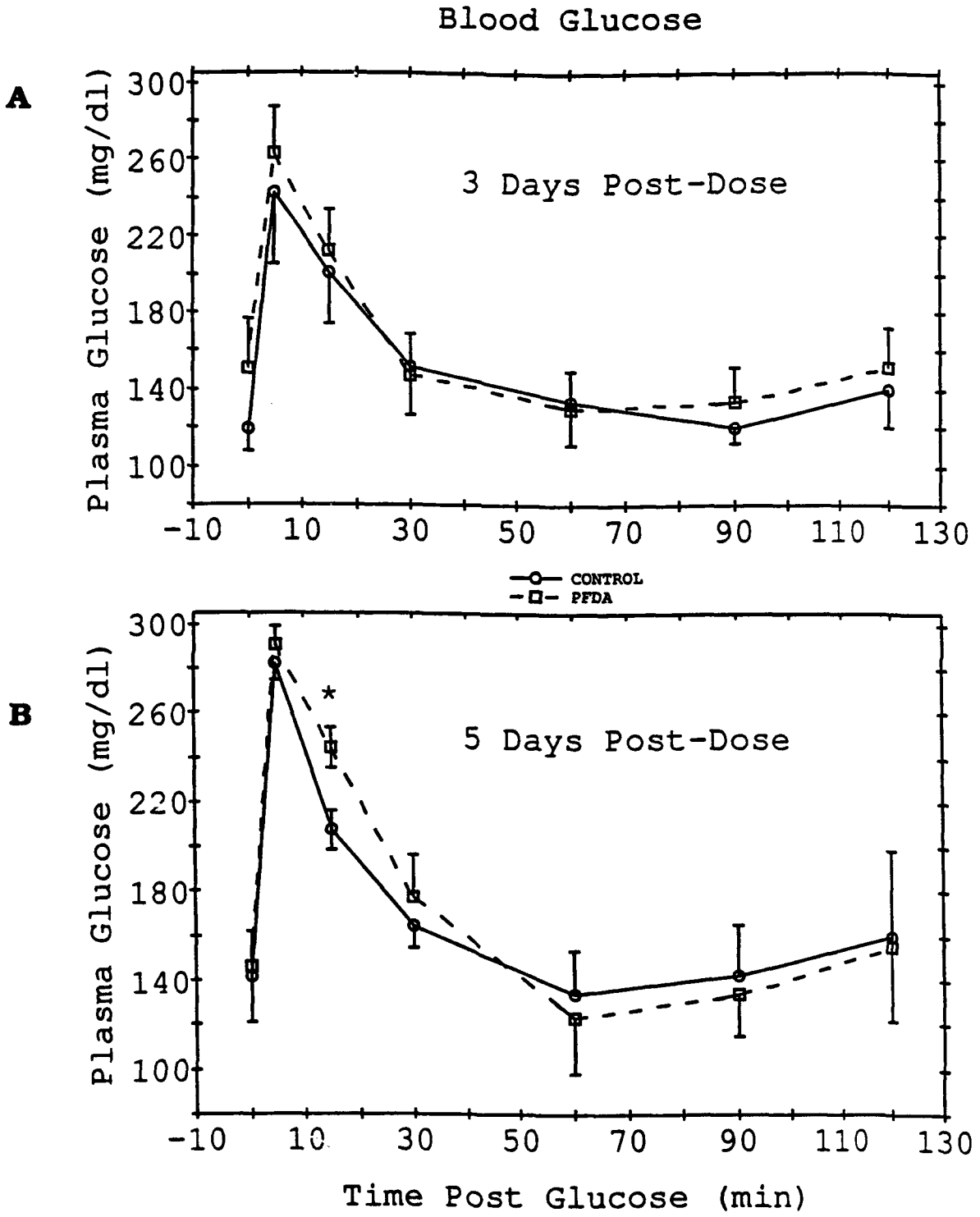

Figure 3: Represents corresponding (mean ± SEM) serum glucose concentrations (mg/dl) in both control and PFDA treated group. (A) Control and FPDA groups at day 3 post treatment (n = 5/group). (B) Control (N = 5) and PFDA (n = 4) groups at day 5 post treatment. *Indicates a significant difference from control.

Hepatic Glycogenesis from Alanine

Experiments are currently in progress which use ^{13}C NMR to monitor hepatic glycogen synthesis from [3- ^{13}C] alanine. Control and PFDA-treated rats are prepared for NMR observation of the liver *in vivo* as described in the previous experiments. While in the NMR magnet, the rats are given a bolus of [3- ^{13}C] alanine (600 mg/kg) via a femoral vein and serial NMR data are acquired. The alanine can be converted to glucose and glycogen through gluconeogenic pathways. This metabolic pathway is independent of glucose transport into hepatocytes and its phosphorylation by glucokinase. Thus data from this study will help to identify the metabolic site of the inhibition in glycogenesis from exogenous glucose.

These results are still preliminary at the time of this writing since the analysis of the data is not yet complete. The data, however, show that gluconeogenesis from alanine is active in control rats and ^{13}C incorporation into hepatic glycogen is clearly evident in all of the control animals (n = 4). The PFDA-treated rats (n=5) also exhibit active gluconeogenesis with incorporation of the ^{13}C label from alanine into hepatic glucose. Surprisingly, one of these animals also displayed glycogen synthesis, although the amount of ^{13}C labeled glycogen is less than that observed in the control animals. It is possible that the PFDA-treated animals have a greater need for the synthesized glucose since normal fatty acid metabolism in these animals is compromised. Thus most of the glucose may be utilized rather than stored as glycogen. Further experiments are in progress in which animals will be given both glucose (unlabeled) and [3-^{13}C] alanine to determine if the ^{13}C label can be incorporated into hepatic glycogen. This protocol will evaluate gluconeogenesis and glycogenesis from alanine in the presence of an exogenous glucose source. Data from this experiment will determine if the pathway from glucose-6-phosphate to glycogen is functional and help to identify the metabolic site of inhibition in glycogenesis from glucose.

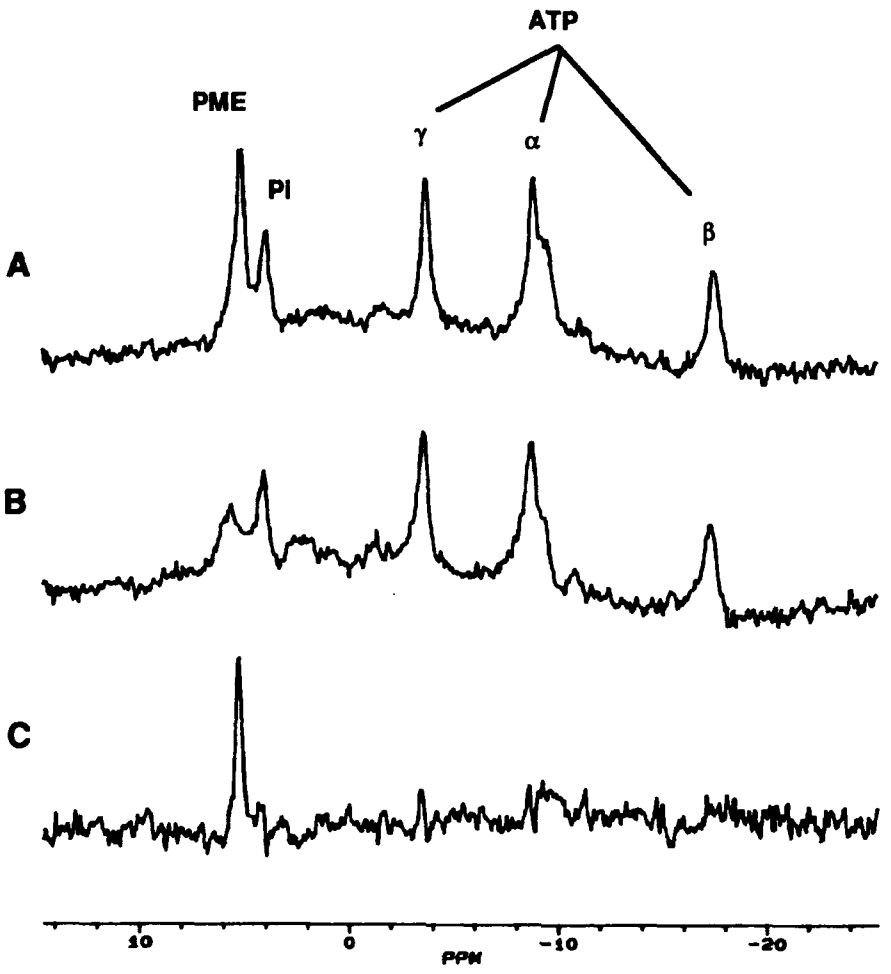

Figure 4: Surface coil ^{31}P NMR spectra of liver *in vivo* at 8.5 T from a PFDA-treated rat at 5 days post dose (A) and its pair-fed control (B). Data were acquired using a 20 μsec pulse (@ 100W), sweep width of 10 kHz, an interpulse delay of 7 sec, and 120 signal averages. The FID's were processed with 2 K total data points and an exponential filter producing a 10 Hz linebroadening. The difference spectrum (C) resulting from A-B depicts the changes which are induced by PFDA. Chemical shifts are referenced to PCr by setting the ß-ATP peak to -16.0 ppm.

Hepatic Phosphorus Metabolism

In another experiment, the high-energy phosphorus metabolites of liver *in vivo* were examined in PFDA-treated rats and their pair-fed controls at 5 days post-exposure. Figure 4 shows the ^{31}P-NMR spectra from a representative pair of animals at 8.5 T. The three signals in the low frequency region are attributed to the α, β, and γ resonances of ATP. Inorganic phosphate (P_i) gives rise to the resonance at ca. 5 ppm. The chemical shift of this resonance is pH sensitive and, thus, can provide a measure of intracellular pH. Figure 4A depicts the spectrum from the PFDA-treated rat and displays a peak of significant intensity in the phosphomonoester (PME) region of the spectrum (ca. 6.4 ppm) as compared to its pair-fed control (Fig. 4B). This increased intensity of the PME signal was observed in all the PFDA-treated rats (n=5). The difference spectrum (Fig. 4C), obtained by subtracting the control spectrum (B) from spectrum (A), clearly displays the increase in the PME signal.

The PME region of the ^{31}P spectrum is believed to be mainly composed of signals arising from phosphorylcholine and phosphorylethanolamine (1-4). An increase in these metabolites is thought to be associated with an increase in membrane synthesis (2). It is interesting to note that previous work has shown that PFDA-treated rats display a proliferation of liver peroxisomes (5). The proliferation of such membrane-bound organelles would require a concomitant increase in membrane synthesis. Thus, peroxisomal proliferation may account for the increased intensity in the PME region of the ^{31}P NMR spectrum. It is also interesting to note that Hayashi and Miwa (6) have suggested that peroxisomes may be involved in the biosynthesis of phospholipids. Further studies will aim to identify this ^{31}P resonance and its biological significance.

The NMR methodology can also be used to monitor changes in ATP levels or intracellular pH as a result of halocarbon treatment. In fact, others have shown a marked decline in hepatic ATP (7) and an intracellular acidosis (8) following treatments with CCl_4 or $BrCCl_3$. Our preliminary data regarding PFDA indicates that a slight decrease in ATP levels and changes in intracellular pH are observed in some animals, but not all. Such effects probably indicate a significant degree of cell necrosis and, perhaps, will be more prominent at later stages of the toxicity. These metabolic effects will be further investigated and will provide a means to evaluate the impact of perfluorocarbon toxicity on overall tissue viability.

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Publications

C.M. Goecke, B.M. Jarnot, and N.V. Reo: "A Comparative Toxicological Investigation of Perfluorocarboxylic Acids in Rats by Fluorine-19 NMR Spectroscopy." *Chemical Research in Toxicology* (Accepted pending appropriate revisions; letter from editor received on December 2, 1991).

Manuscript in Preparation

C.M. Goecke, B.M. Jarnot, and N.V. Reo: "Inhibition of Glucose and Glycogen Metabolism by the Peroxisome Proliferator - Perfluoro-n-decanoic Acid: A Nuclear Magnetic Resonance Investigation *in Vivo*." (Will be submitted to: *Toxicology and Applied Pharmacology*)

Abstracts/Presentations

B.M. Jarnot, C.M. Goecke, and N.V. Reo: "^{31}P NMR of Altered Hepatic Phospholipids Following Exposure to the Peroxisome Proliferator--Perfluor-n-decanoic Acid." Society of Toxicology, Annual Meeting, Seattle, WA, February 1992.

C.M. Goecke, B.M. Jarnot, N.V. Reo: "Effects of Perfluorodecanoic Acid on Hepatic Carbohydrate Metabolism: A ^{13}C NMR Study." Society of Magnetic Resonance in Medicine, Tenth Annual Meting, San Francisco, CA, August 1991.

C.M. Goecke, B.M. Jarnot, N.V. Reo: "A ^{13}C NMR Study of the Effects of Perfluoro-n-decanoic Acid on Hepatic Glycogenesis." Gordon Research Conference on Drug Metabolism, Holderness School, NH, July 1991.

B.M. Jarnot, C.M. Goecke, and N.V. Reo: "Urinary Excretion of Perfluorocarboxylic Acids: A ^{19}F-NMR Investigation." Society of Toxicology, Annual Meeting, Dallas, Texas, February 1991.